Annals of Botany **95**: 773–777, 2005
doi:10.1093/aob/mci087, available online at www.aob.oupjournals.org

Population Genetic Structure of *Monimopetalum chinense* (Celastraceae), an Endangered Endemic Species of Eastern China

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Received: 10 June 2004 Returned for revision: 12 July 2004 Accepted: 22 December 2004 Published electronically: 14 February 2005

• *Background and Aims Monimopetalum chinense* (Celastraceae) standing for the monotypic genus is endemic to eastern China. Its conservation status is vulnerable as most populations are small and isolated. *Monimopetalum chinense* is capable of reproducing both sexually and asexually. The aim of this study was to understand the genetic structure of *M. chinense* and to suggest conservation strategies.
• *Methods* One hundred and ninety individuals from ten populations sampled from the entire distribution area of *M. chinense* were investigated by using inter-simple sequence repeats (ISSR).
• *Key Results* A total of 110 different ISSR bands were generated using ten primers. Low levels of genetic variation were revealed both at the species level ($I_{sp} = 0{\cdot}183$) and at the population level ($I_{pop} = 0{\cdot}083$). High clonal diversity ($D = 0{\cdot}997$) was found, and strong genetic differentiation among populations was detected (49·06 %).
• *Conclusions* Small population size, possible inbreeding, limited gene flow due to short distances of seed dispersal, fragmentation of the once continuous range and subsequent genetic drift, may have contributed to shaping the population genetic structure of the species.

Key words: Celastraceae, endangered species, genetic variation, ISSR, *Monimopetalum chinense*, vegetative reproduction.

INTRODUCTION

Monimopetalum chinense (Celastraceae), the only member of the genus (Rehder, 1926), is a stoloniferous woody vine endemic to eastern China, occurring in the limited range of 28°30′–30°10′N and 114°30′–118°10′E (Xie and Wen, 1999). This species has been subjected to a rapid recent demographic decline, mainly due to habitat destruction, and thus was classified as an endangered species in the *Chinese Plant Red Data Book* (Fu, 1992). Most of the extant populations are small and consist of less than 20 individuals (Xie, 1998). Such small populations are subjected to high risk of extinction or significant loss of genetic diversity. This diploid species ($2n = 20$) can reproduce both sexually and vegetatively. It produces extensive stolons on the ground, from which upright stems and adventitious roots are formed at approx. 1-m intervals, and these upright stems perform sexual reproduction (Xie and Zhang, 1999). Although a detailed study has not been conducted on the reproductive biology of *M. chinense*, a simple spontaneous autogamy test revealed that this species is self-compatible (Xie and Tan, 1998). *Monimopetalum chinense* has a high ovule-abortion rate (57–81 %) and low seed germination rate (<20 %) (Xie and Tan, 1998), and few seedlings were found in indigenous populations.

For plant species capable of reproducing both sexually and asexually, vegetative reproduction is generally expected to have marked effects on the spatial genetic structure of combined asexual and sexual regeneration in plant populations (Chung and Epperson, 2000). Higher rates of asexual reproduction will increase heterozygosity and decrease population differentiation (Balloux *et al*., 2003). As clone structure may reduce the number of genetically distinct individuals within a population, an understanding of clonality is critical for the implementation of the most appropriate conservation management of threatened clonal plants (Young *et al*., 2002), and the same is true for plants with a mixed clonal/sexual breeding system. Due to the mixed vegetative/sexual reproduction character of *M. monimopetalum*, its natural populations potentially encounter a significant proportion of clonal individuals. An understanding of the genetic structure of the natural populations of *M. chinense* is therefore an important prerequisite for effective conservation of the species.

Molecular markers have been widely used to characterize population genetic structure in plants. These markers include allozyme (Widén *et al*., 1994; Guo *et al*., 2003) and polymerase chain reaction (PCR)-based markers like random amplified polymorphic DNA (RAPD) (Gabrielsen and Brochmann, 1998; Kreher *et al*., 2000; Shimizu *et al*., 2002), inter-simple sequence repeats (ISSR) (Hollingsworth *et al*., 1998; Hodkinson *et al*., 2002; Wang *et al*., 2004), amplified fragment length polymorphisms (AFLP) (Escaravage *et al*., 1998; Lamote *et al*., 2002; Albert *et al*., 2003; Isagi *et al*., 2004) and SSR (Reusch *et al*., 2000; Rossetto *et al*., 2004). Inter-simple sequence repeats (ISSR) is a powerful tool for investigating genetic variation within species (Gupta *et al*., 1994; Wolfe and Liston, 1998), especially when sequence information about the study organism is limited. Recent studies on genetic diversity of clonal plant species have demonstrated the great discriminative power of ISSR markers for genet identification

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(Esselman *et al*., 1999; Camacho and Liston, 2001; Li and Ge, 2001; Liston *et al*., 2003; Wang *et al*., 2004).

This paper reports on the use of ISSR in a genetic diversity study of *M. chinense*. The purposes of the investigation are: (*a*) to estimate the extent of clonality and genetic diversity in *M. chinense*; (*b*) to partition the genetic diversity into its within- and between-population components; and (*c*) to propose a conservation strategy based on the observed genetic structure.

MATERIALS AND METHODS

Sampling and DNA extraction

A detailed field survey revealed that the extant *Monimopetalum chinense* Rehd. was restricted to only ten counties in eastern China (Xie and Wen, 1999). One population was sampled from each of these counties. Eight populations were located in Jiangxi Province, and one population in each of Anhui and Hubei Provinces (Table 1 and Fig. 1). Twelve to 20 individuals that do not show clear stolon connections were randomly sampled from each population. In total, 190 individuals were sampled. Genomic DNA was extracted following the CTAB procedure (Doyle, 1991).

ISSR-PCR

One hundred primers of 15–23 nucleotides in length (Biotechnology Laboratory, University of British Columbia, primer set # 9, Vancouver, BC, Canada: http://www.biotech.ubc.ca/services/naps/primers/Primers.pdf) were used to screen for polymorphism. Among them, ten primers (UBC # 808, 810, 811, 835, 841, 857, 876, 880, 889 and 890) yielding polymorphism were used for further study. PCR amplification was carried out in a volume of 20 μL, containing 20 ng of template DNA, 10 mM Tris–HCl (pH 9·0), 50 mM KCl, 0·1 % Triton X-100, 2·5 mM $MgCl_2$, 0·1 mM dNTPs, 2 % formamide, 200 nM primer and 1·5 units of *Taq* polymerase. PCR reactions were performed using a MJ Research 96-well thermal cycler with hot lid following the conditions of Ge *et al*. (2003). PCR products were electrophoresed on 2·0 % agarose gels (10 cm running distance) buffered with 0·5× TBE. A 100-bp DNA Ladder (New England Biolabs) was used as the size marker (100–1000 bp). DNA fragments were characterized by image analysis software for gel documentation (LabWorks Software Version 3.0; UVP, Upland, CA 91786, USA) following staining with ethidium bromide.

Data analysis

Amplified ISSR bands were scored as binary presence or absence characters. Shannon's index of diversity (*I*) (Lewontin, 1972) was calculated using POPGENE v. 1·31 (Yeh *et al*., 1999), as $I = -\Sigma p_i \log_2 p_i$, where p_i is the frequency of a given ISSR fragment. *I* was calculated at two levels: the average diversity within populations (I_{pop}), and the total diversity (I_{sp}). The analysis of molecular variance (AMOVA) was used to partition the total ISSR variation into within-population and among-population components (Excoffier *et al*., 1992). A dendrogram was generated from pairwise genetic distances (F_{ST}) among the populations by the neighbour-joining algorithm using MEGA v. 2·1 (Kumar *et al*., 2001). Under the assumptions of Wright's island model, gene flow (N_m) can be approximated from AMOVA Φ statistics (analogous to *F* statistics) as $N_m = [(1/\Phi_{ST}) - 1]/4$. To test whether genetic distances between pairs of populations were significantly correlated with geographical distances, a Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; Miller, 1997) (computing 5000 permutations).

The clonal diversity was evaluated by the following indices (Ellstrand and Roose, 1987): (*a*) number of genotypes, *G*; (*b*) the mean clone size, $N_c = N/G$, where *N* represents the sample size; (*c*) a modified version of the Simpson diversity index to measure clonal diversity within populations (Ellstrand and Roose, 1987), $D = 1 - \Sigma\{[n_i(n_i - 1)]/[N(N - 1)]\}$; where n_i is the number of samples of genotype *i* and *N* is the total number of the samples.

TABLE 1. *Locations of the sampled populations of* Monimopetalum chinense

Population	Province	Code	Longitude (E)	Latitude (N)
Fengxin	Jiangxi	FX	115°16′	28°49′
Jingan	Jiangxi	JA	115°22′	28°56′
Yongxiu	Jiangxi	YX	115°36′	29°09′
Wuning	Jiangxi	WN	115°22′	29°13′
Tongshan	Hubei	TS	114°40′	29°27′
Yushan	Jiangxi	YS	117°56′	28°52′
Dexing	Jiangxi	DX	117°51′	28°55′
Wuyuan	Jiangxi	WY	117°45′	29°25′
Fuliang	Jiangxi	FL	117°35′	29°33′
Qimen	Anhui	QM	117°29′	29°41′

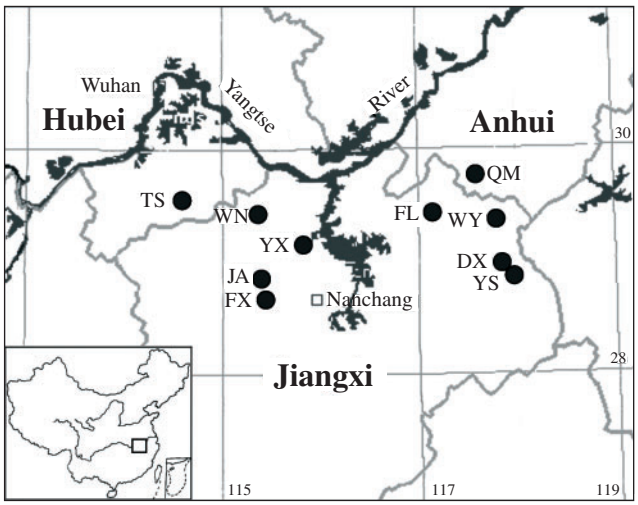

FIG. 1. Map showing locations of the sampled populations of *M. chinense*.

RESULTS

Genetic diversity and structure

A total of 110 different ISSR bands were scored ranging from 200 to 1800 bp, corresponding to an average of

TABLE 2. *Genetic diversity within populations of* M. chinense. *Population codes are given in Table 1*

Population	*N*	I_{pop}	*G*	*N*/*G*	*D*
FX	20	0·095	20	1	1·000
JA	20	0·090	19	1·05	0·995
YX	18	0·101	18	1	1·000
WN	20	0·093	20	1	1·000
TS	20	0·084	18	1·11	0·984
YS	12	0·045	12	1	1·000
DX	20	0·083	20	1	1·000
WY	20	0·081	20	1	1·000
FL	20	0·087	20	1	1·000
QM	20	0·073	19	1·05	0·995
Mean		0·083	18·6	1·02	0·997
		(0·016)	(2·46)	(0·038)	(0·0051)

N, sample size; I_{pop}, Shannon's information index; *G*, number of multilocus genotypes found; *N*/*G*, average clone size; *D*, Simpson's diversity index.

Standard errors are given in parentheses.

TABLE 3. *Analysis of molecular variance (AMOVA) among/within* M. chinense *populations*

Source of variation	d.f.	Sum of squares	Mean squares	Variance component	% of total variance	*P*-value
Among populations	9	69·75	3·62	3·49	49·06	<0·001
Within populations	180	627·73	651·67	3·62	50·94	<0·001

11 bands per primer. Among the 110 loci, 40 were polymorphic at the species level. The Shannon indices ranged from 0·045 to 0·101, with an average of 0·083 ± 0·0156 at the population level (I_{pop}) and 0·183 at the species level (I_{sp}). Among the ten populations, the population from Yongxiu exhibited the greatest level of variability (I_{pop}: 0·101), whereas the population from Yushan showed the lowest level of variability (I_{pop}: 0·045) (Table 2).

There were highly significant ($P < 0·001$) genetic differences among the ten populations of *M. chinense*. Of the total genetic diversity, 49·06 % was distributed among populations and the rest (50·94 %) resided within populations (Table 3). The number of migrants (N_m) was estimated to be 0·26 individuals per generation between populations (Fig. 2). The neighbour-joining dendrogram based on the genetic distance between populations did not show correlations with geographical distance. The Mantel test showed also no significant correlation between genetic and geographic distance ($r = 0·420$, $P = 0·001$).

Clonal diversity

Ten ISSR primers identified 186 genotypes in 190 individuals, and most individuals sampled had a distinct genotype. Over the ten populations, the clone sizes (N_c) ranged from 1 to 1·11, with an average of 1·02. The mean of Simpson diversity index (*D*) was 0·997 (Table 2).

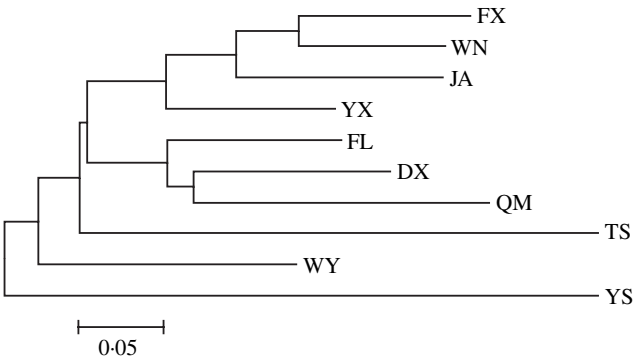

FIG. 2. Neighbour-joining tree of *M. chinense* based on pairwise F_{ST} between populations (for explanation of codes see Table 1).

DISCUSSION

Clonal diversity

Some models regarding the genetic structure of populations with little or no sexual recruitment envision a few localized genotypes, while others consider that asexual populations can be genotypically as polymorphic as sexual ones for plants involving clonal propagation (see Ellstrand and Roose, 1987, and the references therein). Asexual populations maintain higher genetic diversity at each single locus but a lower number of different genotypes. Mixed clonal/sexual reproduction is nearly indistinguishable from strict sexual reproduction as long as the proportion of clonal reproduction is not strongly predominant (Balloux *et al*., 2003). With the increasing application of allozyme and PCR-based DNA markers, it has been found that asexual species often harbour considerable genotypic diversity. For those species with mixed asexual and sexual reproductions, populations usually consist of a number of genets (Ellstrand and Roose, 1987; Eckert and Barrett, 1993; Li and Ge, 2001; Guo *et al*., 2003).

The high levels of clonal diversity in *M. chinense* are somewhat surprising given the facts that this species can propagate through stolons, and seedlings are rare in natural populations (Xie and Zhang, 1999). Every population analysed consisted of numerous genotypes in roughly equivalent frequencies. The clonal diversity in *M. chinense* is higher than that of other clonal plants in general ($D = 0·62$, Ellstand and Roose, 1987; $D = 0·75$, Widén *et al*., 1994), but similar to that of *Adenophora grandiflora* ($D = 0·992$, Chung and Epperson, 1999) and *Viola riviniana* ($D = 0·992$, Auge *et al*., 2001). This result may be partly attributed to the power of ISSR to identify genets (Li and Ge, 2001; Hodkinson *et al*., 2002; Liston *et al*., 2003; Wang *et al*., 2004). Research that compared different markers for clone identification found that ISSR shows much greater variation than allozymes (Esselman *et al*., 1999). Some of the diversity revealed by ISSRs may reflect 'noise' caused by repeatability or lack of homology, a problem inherent to the technique. However, the presence of sexual reproduction is likely to be a more important reason for the high genotypic diversity in *M. chinense*. Despite the low seed set initiation and high embryo abortion (Xie, 1998), *M. chinense*

does produce small amounts of viable seeds. Although the genotypic diversity will decrease at a constant rate with increasing rates of asexual reproduction, a small number of sexual individuals per generation is sufficient to make an asexual population highly genotypically variable (Stehlik and Holderegger, 2000; Balloux *et al*., 2003; Bengtsson, 2003). Computer simulations showed that a single seedling of creeping buttercup (*Ranunculus repens* L.) per generation (about 0·5 % of the total ramet population) was adequate to maintain 15 genotypes (Watkinson and Powell, 1993). In addition, somatic mutations could account, to some extent, for the genetic variation present in clonal populations (Lamote *et al*., 2002). In vegetatively reproducing plants, somatic mutations can be fixed and passed on to the succeeding ramets (Gill *et al*., 1995), and the mutation rates vary across the genet. Unfortunately, mutation rates in *M. chinense* are yet unknown. Finally, the sampling procedure could also have contributed to the high degree of diversity detected. Our leaf samples were taken from the individuals that did not show any stolon connection. To study clone size, more detailed studies aimed at determining the extent of stolon systems using both physical mapping and genetic markers should be carried out in the future.

Genetic diversity and structure

The population genetic diversity and structure of a species is affected by a number of evolutionary factors including mating system, seed dispersal, geographic range as well as natural selection. Of these factors, breeding system is the main one that affects the genetic diversity both among and within populations (Hamrick and Godt, 1990). Inbreeding species tend to have lower levels of genetic diversity and higher levels of differentiation than outcrossing species.

In this study, a low level of genetic variation and a high level of genetic differentiation was detected in *M. chinense* (I_{sp} = 0·183, I_{pop} = 0·083; Φ_{ST}: 49·06 %). Although there have been no comprehensive studies on its breeding system, it was found that *M. chinense* was self-compatible and it could produce viable seeds through selfing (Xie and Tan, 1998). Possible inbreeding may be one of the most significant determinants of the low levels of genetic diversity within populations and relatively high levels of genetic differentiation among populations in this species. Lack of effective mechanisms for long-distance dispersal of seeds may also play an important role in shaping the observed genetic structure (Wallace, 2002). For *M. chinense*, an indirect estimate of the number of migrants per generation (N_m = 0·26) was less than one. The levels of gene flow calculated here are of insufficient magnitude to counter-balance genetic drift that may also play a role in the observed population differentiation.

The limited range of distribution was another important reason for the observed genetic pattern in *M. chinense*. Species with restricted distribution ranges usually have lower genetic diversity, whereas species with discrete populations in a patchy distribution have lower levels of variation within populations than species with more continuous distributions. The natural distribution of *M. chinense* is restricted to a small area of the lower reaches of the Yangtse River. Coupled with the increased destruction of the broadleaf forest below 1000 m a.s.l. where *M. chinense* grows, its populations are subject to fragmentation. About 73 % of populations are fewer than 20 individuals based on the analysis of 141 populations at its centre of distribution (Xie, 1998). Two major genetic consequences of small-population size for long periods of time are high levels of genetic drift and inbreeding (Barrett and Kohn, 1991; Ellstrand and Elam, 1993). Both of these factors could be responsible for the genetic structure of this species. The Mantel test and the neighbour-joining dendrogram (Fig. 2) provided further support for the conclusions as there is no significant correlation between genetic distance and geographical distance.

Conservation strategies

It is critical to recognize the extent of clonality in threatened species, in order to choose the relevant strategy for conservation management (Sydes and Peakall, 1998). This study has revealed very high clonal diversity in *M. chinense*. Provided that determination of genotypes has not been compromised by technical features of ISSR, almost every plant that has no stolon connection belongs to a different genet. Therefore, the effective size of the populations could be counted directly. Based on the low level of genetic diversity in *M. chinense*, the most suitable strategy for its conservation is the protection of its habitat. The observed strong genetic differentiation among populations of *M. chinense* indicates that management for conservation of genetic variability in *M. chinense* should not only aim to preserve large populations but also as many of the small populations as possible. For *ex situ* conservation of *M. chinense*, because of its low germination rate, growing new plants from cuttings or tissue culture are suitable options for transplantation.

ACKNOWLEDGEMENTS

This study was financially supported by the Key Project of the Chinese Academy Sciences (KSCX2-SW-104) and National Natural Science Foundation of China (grant no. 30470146).

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